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FOREWORD

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I. INTRODUCTION

A. Nature of the Problem

Breast cancer is the leading cause of cancer-related death among women in this country and the incidence of breast cancer among young women is rising. Although early detection techniques (mammography) are available, early detection has not led to a decline in mortality rates and cannot be used to prevent or treat breast cancer. Developing new strategies to improve breast cancer survival will depend upon a greater understanding of the pathogenesis of breast cancer and the events which lead to neoplastic transformation of breast epithelial cells.

Cancer cells differ from normal cells in that they undergo uncontrolled cell growth and acquire the abilities to invade adjacent tissues, enter the circulatory system, and "home" to sites distal from the primary tumor (metastasis). An important aspect of tumor invasion and metastasis involves the adhesive interactions of tumor cells with other cells or the extracellular matrix. Many of these adhesive interactions are mediated by the integrin family of cell adhesion receptors (reviewed by Hynes, 1992). Integrins provide not only a structural means of cell anchorage but also a means of transmitting signals regulating gene expression and protein function (Clark and Brugge, 1995). It is possible that extracellular matrix proteins regulate normal cell growth by transmitting signals to the interior via specific integrin receptors. In transformed cells, such signaling pathways might be absent or dysfunctional thereby conferring unresponsiveness to normal growth constraints. **Our efforts are focused to understand how integrins participate in the regulation of cell division in normal breast cells and to determine how breast cancer cells escape these regulatory pathways.**

B. Background of Previous Work

1. Integrins. Integrins are transmembrane glycoproteins comprised of two non-covalently associated subunits (α and β) that mediate both cell-cell and cell-substrate adhesion. Integrin receptors bind extracellular matrix (ECM) and plasma proteins, non-integrin adhesion receptors and other integrins (reviewed by Hemler, 1990; Hynes, 1992). In addition to their role as primary mediators of cell adhesion, it has now become clear that integrins are also capable of transducing signals to the cell interior (Hynes, 1992, Ginsberg et al, 1992, Juliano, 1993, Juliano, 1994, Clark and Brugge, 1995). Tyrosine phosphorylation appears to be a key aspect of integrin mediated signal transduction and a tyrosine kinase (p125 FAK) has been identified which localizes to focal adhesions when cells adhere to an ECM protein-coated surface (Schaller et al, 1992). In addition, evidence is accumulating which suggests that integrin-mediated signaling events can induce gene expression (Yurochko et al, 1992) and affect transit through the cell cycle (Matsuyama et al, 1989, Symington, B.E., 1992, Mortarini et al, 1992). Many of the ECM proteins that serve as ligands for integrins have been identified and include: fibronectin (FN), vitronectin (VN), laminins, and collagens. Some integrins can interact with more than one ligand (e.g. $\alpha 4 \beta 1$, $\alpha v \beta 3$, $\alpha 3 \beta 1$) while several ECM

proteins are recognized by more than one integrin. For example, FN has been shown to interact with multiple integrin receptors ($\alpha 5\beta 1$, $\alpha 4\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 6$, $\alpha v\beta 1$, $\alpha 3\beta 1$)

2. Cell cycle Regulation. The initiation of cell division and transitions between different stages of the cell cycle involves signals which activate the association of specific protein complexes (cell cycle dependent kinases (CDK) with the regulatory cyclins). At each checkpoint certain cyclins and CDKs form active complexes which phosphorylate and thereby activate specific proteins necessary for DNA replication (G1/S), mitosis, and cytokinesis (G2/M). Recently, it has been shown that the activity of cyclin/CDK complexes is regulated by a family of CDK inhibitor proteins, referred to as CDIs (p16, p21, p27) that bind to and inactivate the CDKs (Hunter and Pines, 1994). **The purpose of this project is to determine how signals transduced via integrin receptors, especially $\alpha 5\beta 1$, regulate the formation of CDK/cyclin complexes in neoplastic and normal breast cells.**

Previous results suggested that engagement of a fibronectin receptor, the integrin $\alpha 5\beta 1$, with its fibronectin peptide ligand, Gly-Arg-Gly-Asp-Ser (GRGDS), affects CDC2/cyclin A complexes in partially transformed but not fully transformed breast cancer cell lines (Symington, B.E., 1995). Since normal and neoplastic breast epithelial cells express multiple integrins which bind different ECM proteins, we are interested in expanding our studies to other integrin-ECM interactions. In addition, we will examine the contribution of these receptors in several *in vitro* assays relevant to metastasis and homing. Understanding the mechanisms involved in these processes may permit development of therapeutics which will inhibit the growth or metastasis of breast cancer cells.

C. Purpose of the Present Work

During the past year, we have focused on expanding the initial breast cancer observations. That is, what role does $\alpha 5\beta 1$ play in the regulation of cell proliferation of normal mammary epithelial cells and breast cancer cells? In order to understand the role of integrins in tumor cell biology, it is important to have information concerning the pattern of integrin expression in transformed cells and to ask whether there is any relationship between integrin expression and tumor progression. Therefore, our approach has been to:

- 1). Expand the initial studies with RGD (GRGDS) peptides to other breast cancer cell lines and to normal primary mammary epithelial cells. Characterize the expression of integrins on each cell line and normal cells by flow cytometry.
- 2). Examine the expression of integrins in normal and tumor tissue using immunohistochemistry.
- 3). Isolate primary cells from both normal and cancer breast tissue to determine their profile of integrin expression and cell cycle kinase response to RGD peptides.

II. BODY

A. Methods

1. Patient Samples

Tissue specimens were obtained from lumpectomy and mastectomy tissues provided by Dr. Ron Tickman who is a staff pathologist in the Laboratory of Pathology, Swedish Hospital Medical Center, Seattle, WA and used for immunohistochemistry and the isolation of breast cells (BC). These samples were removed from patients undergoing surgery at Swedish Hospital solely for diagnostic or therapeutic purposes and would otherwise be discarded. Information identifying the patients was withheld in order to comply with the requirements to obtain exemption status from complete IRB review. However, we have access to information concerning the patient's age and sex, tumor histology, estrogen and progesterone receptor status, and the presence and location of metastases. This will enable me to correlate tumor type, malignancy, integrin expression and response to $\alpha 5 \beta 1$ ligation. Although no patient samples will be excluded on the basis of race, age, sex, religion, or ethnic background, the low incidence of male breast cancer will preclude collection of a large number of samples from men. This use is approved by the IRB.

2. Monoclonal and Polyclonal Antibodies

Anti-integrin Monoclonal Antibodies. Several monoclonal antibodies (Mabs) directed to a variety of integrin receptors expressed by normal and neoplastic epithelial cells have been produced, previously (Wayner and Carter, 1987; Wayner et al, 1988; Carter et al., 1990a, 1990b; Brown et al, 1991; Wayner et al, 1991; Wayner et al, 1993). Many of these Mabs perturb integrin function and will be used to determine how normal and neoplastic breast cells interact with FN or GRGDS peptide. These Mabs include the two used by Dr. Symington to ligate $\alpha 5$ or $\beta 1$ (P1D6 (anti- $\alpha 5$) and P4C10 (anti- $\beta 1$) as well as inhibitory antibodies P1H5 (anti- $\alpha 2$), P1B5 ($\alpha 3$), P4C2 ($\alpha 4$), P5H9 ($\alpha v \beta 5$), and LM609 ($\alpha v \beta 3$, courtesy of Dr. David Cheresh, The Scripps Research Institute, La Jolla, CA).

Antibodies Directed to Human Cell Cycle Proteins. Monoclonal or polyclonal antibodies directed to human CDKs (CDC2, CDK2-CDK5), cyclins (A, B1, D1-D3, E), CDIs (p16, p21, p27), and tumor suppressor proteins (Rb and p53) were obtained from Pharmingen (San Diego, CA). The Mab to breast cancer antigen CA15-3 was obtained from Chemicon Intl. (Temecula, CA). E-cadherin was detected with Mab HECD-1 (courtesy of M. Takeichi).

3. Breast Cell Isolation and Culture

Normal human BC was obtained from Clonetics Corp (San Diego, CA, cat. # CC-0228) or from reduction mammoplasty tissue supplied by Dr. Ron Tickman. Breast epithelial cells were isolated according to published protocols (Taylor-Papadimitriou and Stampfer,

1992; Blaschke, R.J. et al., 1994, Bergstraesser L.M and Weitzman, S.A., 1993). Briefly, the samples were minced and enzymatically digested to isolate mammary glands and single cells. After an initial plating in serum-containing medium, cells were cultured in serum-free mammary epithelial growth medium (MEGM) from Clonetics (cat. #CC-3051). A number of breast carcinoma cell lines (see Table I) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were cultured as recommended by the ATCC.

4. Flow Cytometry Analysis

Flow cytometry analysis was used to identify the integrin receptors expressed by cultured normal and transformed BC lines. Cells were incubated with anti-integrin Mabs (10 $\mu\text{g/ml}$ or culture supernatant) for 30 min in suspension in FACS buffer (HBSS supplemented with 1% goat serum and 0.02% sodium azide). Cells were then washed and incubated with affinity purified FITC-conjugated goat anti-mouse, 2 $\mu\text{g/ml}$ (Southern Biotechnology, Birmingham, AL). The appropriate isotype matched controls were included with each sample. Cells were analyzed by forward light scatter (linear) versus green fluorescence (log). Flow cytometric analysis of stained cells were performed on either an Coulter EPICS C System or a Becton Dickinson FACScan equipped with an argon laser. At least 5,000 events were analyzed for each anti-integrin antibody and compared to a matched isotype control.

5. Immunohistochemistry

Normal and tumor breast tissue were obtained from Dr. Ron Tickman, embedded in Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN), and frozen on a block of dry ice. Tissue sections (6-10 microns) were cut using an IEC cryostat. Sections were analyzed for expression of integrins and extracellular matrix proteins using monoclonal antibodies as described by Hoffstrom and Wayner (1994).

6. Kinase Assay

The *in vitro* kinase assay was performed essentially as described (Symington, B.E., 1992). Briefly, normal or breast cancer cell lines were incubated for 2 h in culture media alone or in media containing GRGDS (or GRGES) peptides (10 μg per 5×10^5 cells). Cell lysates were prepared and reacted with anti-CDC2, anti-CDK2, anti-cyclin A, anti-cyclin E, or normal rabbit serum followed by protein A-agarose beads. Protein A beads were washed and resuspended in kinase buffer (20 mM Tris pH 6.8, 10 mM MgCl_2 , 1 mM dithiothreitol, 30 μM ATP, 10 mCi of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ (3000 Ci/mmol; Amersham Corp) with or without 1 μg histone H1 per reaction tube and incubated at 37° C for 30 min prior to solubilization in SDS-PAGE sample buffer and electrophoresis on 12% polyacrylamide slab gels. Gels were stained with Coomassie brilliant blue, dried, and exposed to film.

B. Results and Discussion

1. Integrin expression and effects of RGD (GRGDS) peptides on cell cycle kinase activity in normal mammary and breast carcinoma cell lines.

So far, we have characterized 12 different breast carcinoma cell lines as well as on one batch of normal mammary epithelial cells for expression of the integrins $\alpha 5$, $\alpha 3$, $\alpha 4$, $\beta 1$, $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha 6$, $\beta 4$, and $\alpha 2$ (Table I). We've also included E-cadherin and CA 15-3 as potential markers for normal epithelial cells and cancerous cells (Table I). Of these integrins, $\alpha 3 \beta 1$ was the only integrin expressed at significant levels in nearly all of the cell lines and it was most strongly expressed by normal cells. Most cell lines expressed low levels or were negative for $\alpha 5$, $\alpha 6$, $\beta 4$, and $\alpha v \beta 3$. Intermediate, but variable expression was observed for $\alpha 2$ and $\alpha v \beta 5$. HMEC expressed intermediate levels of $\alpha 5$ and $\alpha 4$, low levels of $\alpha 6$ and $\alpha v \beta 5$, and were negative for $\alpha v \beta 3$ and $\beta 4$. Although normal cells expressed E-cadherin but not CA15-3, these markers, by themselves, do not distinguish normal from tumor cells since most of the carcinomas expressed E-cadherin and a few did not express CA15-3.

Initial experiments by Dr. Symington suggested that regulation of CDC2 kinase activity by RGD peptides occurred only in non-transformed cells and that fully transformed lines had a defect in this pathway. This hypothesis was based on a comparison of two breast cancer cell lines (HBL 100 and BT20) as well as normal human epidermal keratinocytes with two virally transformed keratinocyte cell lines (Symington, B.E., 1995).

During this past year, we have extended these studies to 5 additional breast cancer cell lines (MDA-MB-453, MDA-MB-157, DU 4475, T47D, and MCF7) and to normal primary mammary epithelial cells (HMEC). One might expect that T-47D (partially transformed, agar growth) and MDA-MB-453 (non-tumorigenic, nude mice) but not the tumorigenic cell lines MDA-MB-157, DU 4475, or MCF7 would increase CDC2 kinase activity in response to soluble RGD peptides. While a slight increase (2-fold) of CDC2 kinase activity was observed in MDA-MB-453 cells, RGD peptides had no effect on CDC2 kinase activity in T-47D cells (Fig 1). This is despite the fact that T-47D cells express the RGD-binding integrins $\alpha 5 \beta 1$ and $\alpha v \beta 5$, but not $\alpha v \beta 3$ (Table I). However, it should be noted that T-47D cells are tumorigenic in the presence of estrogen and pituitary factors (Leung, C.K and Shiu, R.P., 1981). Since MDA-MB-453 cells express $\alpha v \beta 5$, but not $\alpha 5 \beta 1$ nor $\alpha v \beta 3$, the stimulation of CDC2 kinase activity appears to be mediated by RGD peptides binding to an integrin other than $\alpha 5 \beta 1$, possibly $\alpha v \beta 5$, $\alpha 3 \beta 1$, or other, yet to be identified, integrin(s). In the tumorigenic cell lines, RGD peptides had no effect (DU-4475, MCF7) or produced a slight increase (1.5 fold, MDA-MB-157) in CDC kinase activity. Thus, RGD peptides may be capable of regulating cell growth in MDA-MB-157 cells. The lack of an effect in DU-4475 cells may be explained by the low level of CDC2 kinase activity or by the absence of the integrins $\alpha 5 \beta 1$, $\alpha v \beta 3$, and $\alpha v \beta 5$. However, an increase in cyclin A associated kinase activity was observed in these cells suggesting that RGD peptides are binding to an integrin(s). Interpretation of the MCF7 data may not be reliable because there are two populations of cells, one that is

negative for E-cadherin and one that is positive (Table I). We are in the process of cloning out these two populations of cells and will repeat these studies with the cloned populations. MDA-MB-157 cells express low levels of $\alpha 5\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$. The integrin $\alpha 3\beta 1$ may be the most prominent integrin expressed by MDA-MB-157 cells, although our characterization of integrin expression is incomplete.

Thus, the regulation of CDC2 kinase activity by RGD peptides does not appear to be limited to the integrin $\alpha 5\beta 1$ and also may not correspond with the tumorigenic potential of the cell. Moreover, in normal mammary epithelial cells, RGD peptides failed to stimulate CDC2 kinase activity even though this batch of primary cells expressed the integrin $\alpha 5\beta 1$, low levels of $\alpha v\beta 5$, and no $\alpha v\beta 3$ (Fig 1 and Table I). It is possible that $\alpha v\beta 5$ or other integrins (such as $\alpha 3\beta 1$ or other αv -integrins, possibly $\alpha v\beta 6$, $\alpha v\beta 1$) on normal mammary epithelial cells have a higher affinity for soluble RGD peptides than $\alpha 5\beta 1$ but that these other integrins do not regulate CDC2 kinase activity. Such a scenario casts doubts on the relevance of mediating cell proliferation through low valence interactions between the integrin $\alpha 5\beta 1$ and RGD-containing fragments of ECM proteins *in vivo*. Furthermore, these results illustrate the complex nature of working with cells expressing multiple integrins which can bind RGD peptides rather than the cell line FA-K562 which was used for the initial studies (Symington, B.E., 1992) and which appeared to express only $\alpha 5\beta 1$ as its RGD-binding integrin.

In addition to CDC2, we also assayed these cell lines for cyclin A and cyclin E associated kinase activity. Both cyclin A and cyclin E have been implicated in the anchorage-dependent growth of fibroblasts (Symington, B.E., 1995; Guadagno, T.M. et al., 1993, Zhu, X. et al., 1996). We have observed both increases (HMEC, DU-4475, T47D, MCF7) and decreases (MDA-MB-453 and MDA-MB-157) in cyclin A associated kinase activity (Fig 2). These changes did not correspond with the effects on CDC2 kinase activity in the same lysate suggesting that RGD peptides were not affecting cyclinA/CDC2 complex formation. We also examined CDK2 in MDA-MB-157 and MDA-MB 453 cell lysates and did not observe any effects on kinase activity. No effects were observed on cyclin E associated kinase activity. These results suggest that, in mammary epithelial cells, integrins may regulate a cyclin A/CDK interaction other than cyclin A with CDC2 or cyclin A with CDK2. However, we need to investigate this further through co-immunoprecipitation/Western blot methods. This regulation does not appear to be correlated with either expression of a specific integrin or tumorigenic potential.

2. Immunohistochemistry of normal and cancerous breast tissue. Integrin expression in normal mammary breast tissue.

Culture conditions (FBS, growth factors) of cells may alter their phenotype resulting in changes in integrin expression. An example of this is the induction of the integrin $\alpha 5\beta 1$ the long-term (10-15 passages) of normal, primary keratinocytes. In order to evaluate the potential role of the integrin $\alpha 5\beta 1$ in normal mammary epithelium, we analyzed tissue from several different donors for the expression of $\alpha 5\beta 1$ and other integrins by

immunohistochemistry (IHC). To date, we have not detected significant levels of $\alpha 5 \beta 1$ in either basal or ductal mammary epithelial cells. Fig 3, panel A shows the immunostaining of a normal breast tissue with the anti- $\alpha 5$ monoclonal antibody, P1D6. Several stromal cells (fibroblasts) adjacent to the duct stained positive with this antibody while the ductal epithelial cells were negative. This is in contrast to the staining for the integrin $\alpha 3$ with the monoclonal antibody P1B5 in which both the ductal epithelium and stromal fibroblasts were strongly positive (Fig 3, panel C). Occasionally, some epithelial cells appeared positive for $\alpha 5 \beta 1$ which may represent a specific sub-population of mammary epithelial cells, perhaps those retaining full proliferative potential. Alternatively, $\alpha 5 \beta 1$ expression may be regulated locally by extracellular factors such as cytokines or growth factors. Another fibronectin receptor, $\alpha 4 \beta 1$, also was not expressed by mammary epithelial cells. From our IHC results, normal mammary epithelium consistently stained positive for the integrins $\alpha 3 \beta 1$, $\alpha 6 \beta 4$, $\alpha 2 \beta 1$, and $\alpha 1 \beta 1$. The expression of αv integrins varied from one specimen to the next and sometimes within the same specimen. This variability was observed for all of the αv integrins examined (αv subunit, $\alpha v \beta 3$, $\alpha v \beta 5$, and $\beta 6$). In general, basal (myoepithelial) cells expressed higher levels of integrins than the ductal cells.

In tumor specimens, tumor cells were identified by their expression of the breast carcinoma-associated antigen, CA 15-3. In normal ducts, CA 15-3 is localized to the luminal surface of ductal epithelial cells while a disperse pattern of expression is observed in cancer cells (Fig 4 panels A and B). Of the integrins expressed in normal mammary ducts, $\alpha 3 \beta 1$ was the only one which was consistently expressed by tumor cells, both within ducts and those infiltrating the adjacent stroma (Fig 4, panel D). The expression of integrins was, in general, higher in the tumor cells present within ducts than in the infiltrating population. The basement membrane protein, laminin 5, was abundant in normal mammary ducts and usually present in cancerous ducts, but at sometimes reduced levels. Infiltrating tumor cells usually did not express laminin 5, or, when present, was not organized into a basement membrane structure (Fig 4, panels E and F). Integrin $\beta 4$ expression was either diminished or absent in infiltrating carcinomas (Fig 4, panels G and H). These observations suggest that expression and organization of laminin 5 and the integrin $\beta 4$ may be crucial to the maintenance of normal gland structures and that loss or aberrant expression of these components contribute to the infiltrating phenotype of breast cancer cells. These observations are also reflected in the breast cancer cell lines. Whereas laminin 5 is easily detected in the conditioned medium from normal mammary epithelial cells, laminin 5 has not been detected in the conditioned medium from any of the breast cancer cell lines. Also, the lack of expression of the $\beta 4$ integrin subunit by the breast carcinoma cell lines is consistent with the expression of $\beta 4$ by infiltrating tumor cells.

3. Isolation and characterization of primary cells from normal and tumor tissue.

A great deal of effort has also been placed into isolating epithelial cells from both normal and cancerous breast tissue. We have gotten both epithelial and fibroblast cells to grow from these specimens. However, we have been unable to isolate enriched cell

populations in quantities sufficient for either cell cycle kinase experiments or flow cytometric analysis. Nearly all of the tumor specimens have been very small in mass due to the use of mammography and other early detection techniques (breast examination) making cell isolation even more difficult. Efforts to characterize the cells by immunohistochemistry for cytoskeleton components (cytokeratins, alpha-smooth muscle actin, vimentin), integrin expression, and expression of CA15-3 have been hindered by high non-specific staining. Analysis of the culture supernatant for laminin 5 and fibronectin appears to be useful for distinguishing normal mammary epithelial cells from fibroblasts. That is, normal mammary epithelial cells express both laminin 5 and fibronectin while fibroblasts express only fibronectin. It is unclear whether primary tumor cells express laminin 5. Immunostaining of tissue sections indicates that infiltrating tumor cells do not consistently express laminin 5. However, we have yet to isolate a culture with a clearly epithelial morphology which also does not express laminin 5. Another means of characterizing the primary cells is their growth within a three-dimensional Matrigel matrix. Matrigel induces the formation of gland-like structures by normal mammary epithelial cells while tumor cells grow as colonies (Peterson O.W., et al., 1992). We have recently incorporated this system into our studies and have observed the formation of gland-like structures, presumably arising from normal mammary epithelial cells. We are also attempting to isolate these gland-like structures from the Matrigel as a means of enriching for normal mammary epithelial cells.

Table I											
Relative level of expression based on comparison of FACS histograms.											
Cell line	E-Cadherin	CA15-3	alpha5	alphaVbeta3	alphaVbeta5	alpha3	alpha 4	alpha 6	beta 1	beta 4	alpha 2
HMEC	(+++)	(-)	(++)	(-)	(+)	(++++)	(++)	(+)	(+++)	(-)	ND
HBL 100	ND	ND	(++)	(+)	(+)	(+++)	(++)	(+)	(++)	(-)	ND
BT20	ND	ND	(++)	(+)	(+)	(-)/(+++)	(++)	(+)	(-)/(+++)	(-)	ND
T47D	(+++)	(++)	(+)	(-)	(++)	(++)	(+)	(+)	(++)	(-)	ND
DU4475	(++)	(+++)	(-)	(-)	(-)	(+)	(-)	(++)	(++)	(-)	ND
MDA-MB-453	(-)	(-)	(-)	(-)	(+)	(++)	(-)	(+)	(++)	(-)	ND
MDA-MB 157	(-)	(++)	(+)	(+)	(+)	(+++)	(+)	(-)	(++)	(-)	(-)
BT 483	(-)/(+++)	(++)	(+)	(+)	(++)	(++)	(+)	(+)	(++)	(-)	(++)
Hs578t	(-)	(++)	(++)	(+)	(++)	(++)	(++)	(-)	(++)	(-)	(++)
MDA-MB 361	(++)	(+)	(+)	(-)	(+)	(++)	(+)	(+)	(++)	(-)	(+)
MCF7	(-)/(+++)	(+++)	(-)	(-)	(++)	(+)	(-)	(-)	(++)	(-)	(++)
BT474	(++)	(++)	(-)	(-)	(+)	(++)	(-)	(+)	(+)	(-)	(+)
MDA-MB-134-VI	(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(-)

Table I. Integrin expression on normal mammary epithelial cells (HMEC) and breast carcinoma cell lines. Cell surface expression was assayed by flow cytometry using monoclonal antibodies to specific integrin subunits or complex-specific ($\alpha\beta 3$, $\alpha\beta 5$) antibodies. Resulting histograms were scored as either negative (-) or positive (+ to +++) by comparison against normal IgG or no antibody controls. Two scores within the same box separated by a diagonal line indicates the detection of 2 cell populations. ND= not determined.

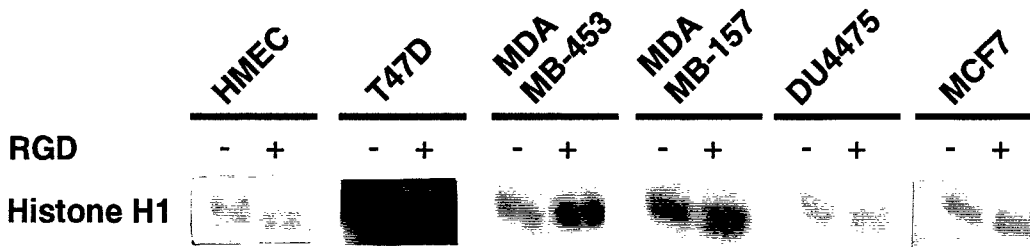


Fig. 1. CDC 2 Kinase Activity. Normal mammary epithelial cells (HMEC), 2 partially transformed cell lines (T47D, MDA-MB-453) and 3 tumorigenic cell lines (MDA-MB-157, DU4475, and MCF-7) were incubated with (+) or without (-) RGD peptides for 2 hours. CDC 2 was immunoprecipitated from cell lysates and assayed for kinase activity using histone H1 and $[^{32}\text{P}]\text{-}\gamma\text{-ATP}$ as substrates. Reaction products were separated by SDS-PAGE and phosphorylated histone H1 was detected by autoradiography.

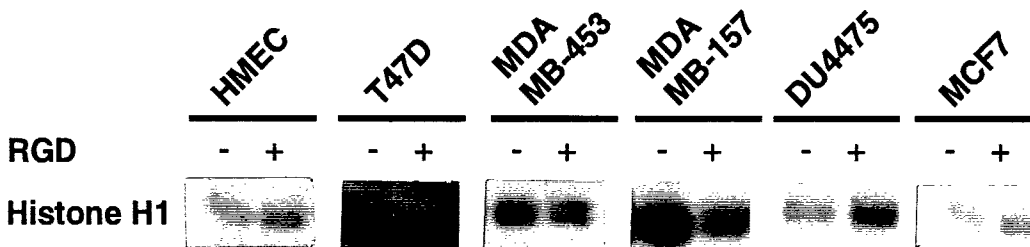


Fig. 2. Cyclin A associated Kinase Activity. Normal mammary epithelial cells (HMEC), 2 partially transformed cell lines (T47D, MDA-MB-453) and 3 tumorigenic cell lines (MDA-MB-157, DU4475, and MCF-7) were incubated with (+) or without (-) RGD peptides for 2 hours. Cyclin A was immunoprecipitated from cell lysates and assayed for kinase activity using histone H1 and $[^{32}\text{P}]\text{-}\gamma\text{-ATP}$ as substrates. Reaction products were separated by SDS-PAGE and phosphorylated histone H1 was detected by autoradiography.

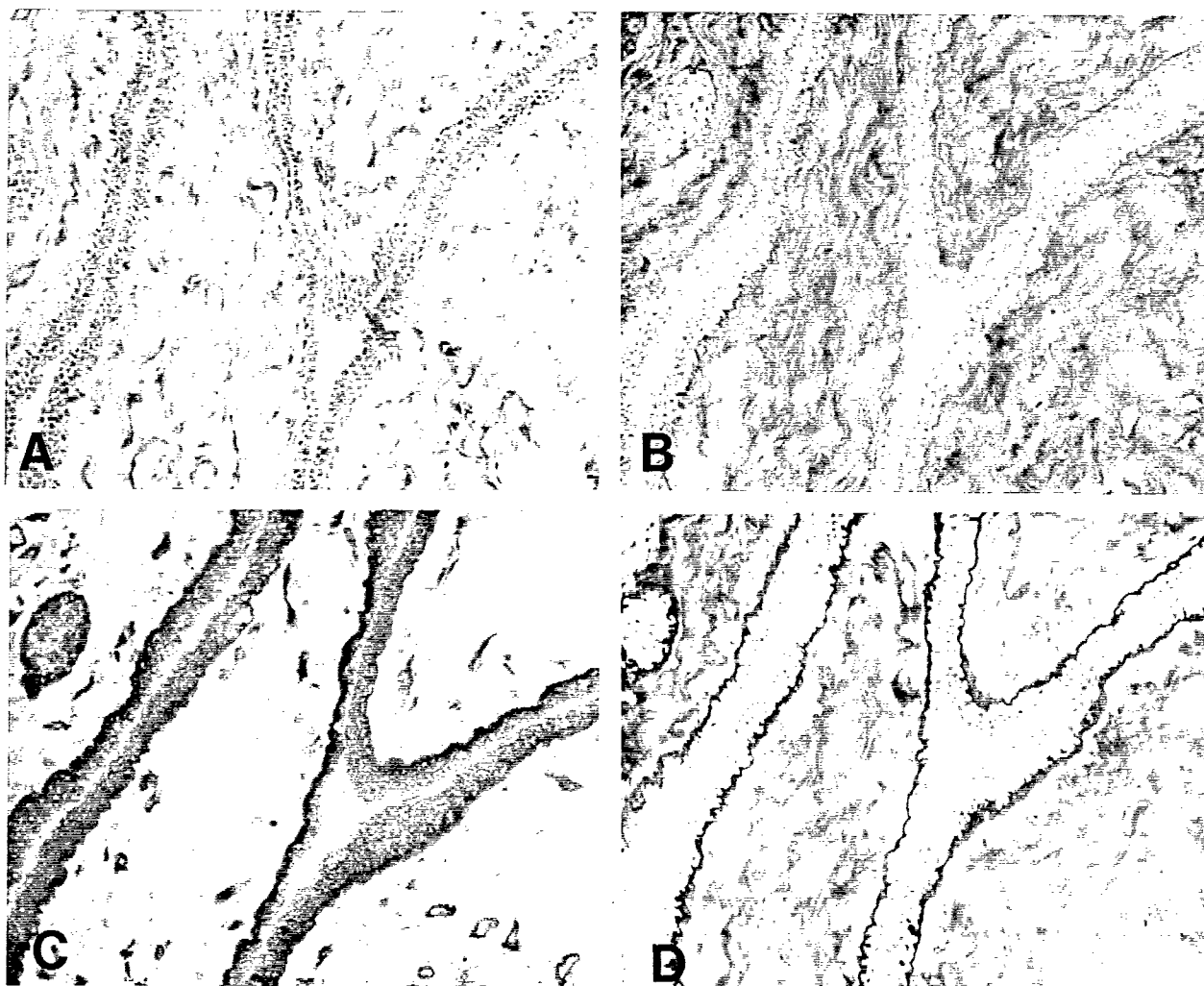
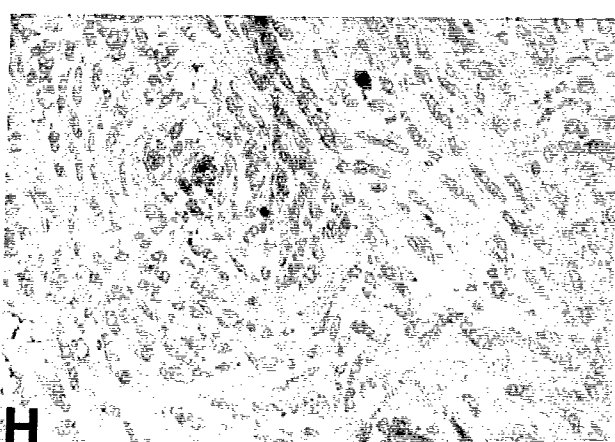
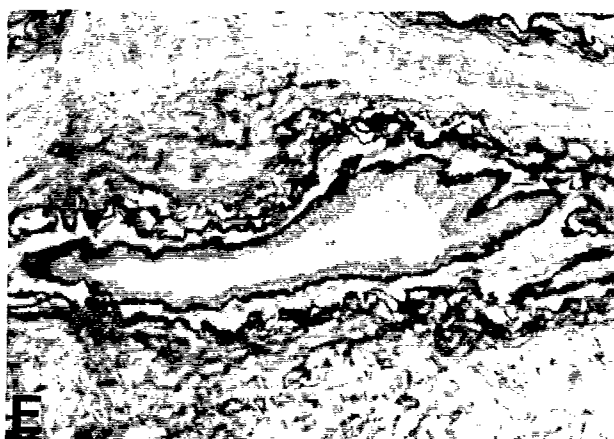
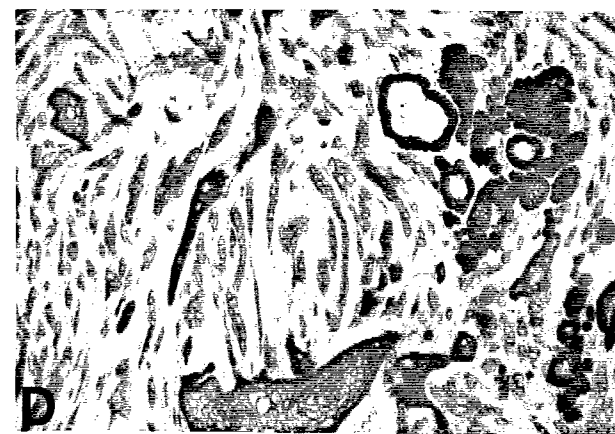
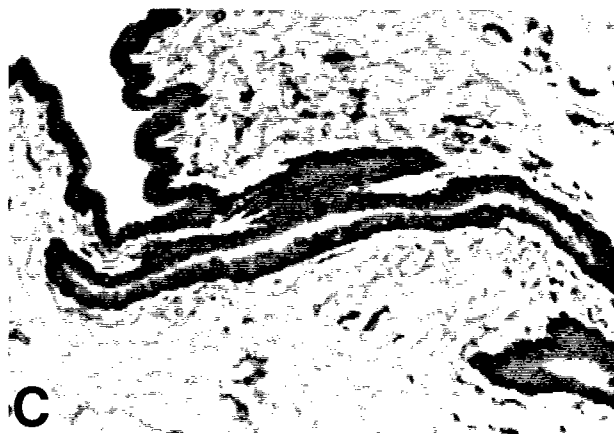
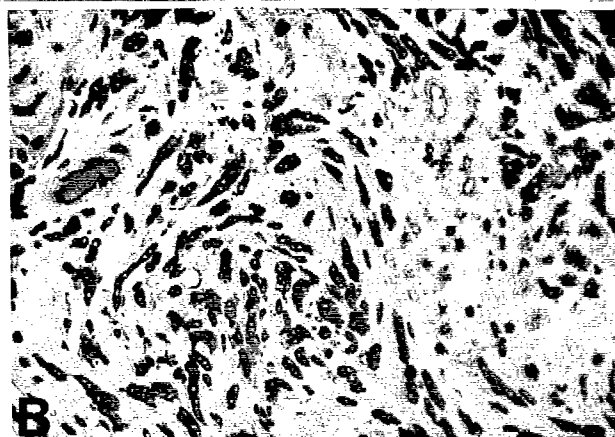
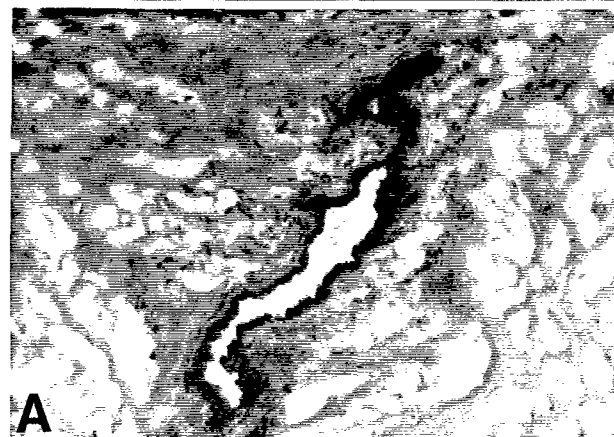


Fig 3. Normal mammary epithelium does not express the fibronectin receptor $\alpha 5\beta 1$.

Frozen sections of normal human breast were incubated with monoclonal antibodies to integrins alpha 5 (panel A) or alpha 3 (panel C) and their respective ligands, fibronectin (panel B) or laminin 5 (panel D). Bound antibodies were visualized as described in Methods and sections were counterstained with hematoxylin.



UNPUBLISHED DATA

Fig 4. Infiltrating carcinoma cells express $\alpha 3\beta 1$ but little $\alpha 6\beta 4$ or laminin5. Frozen sections from normal (panels A,C,E,G) or infiltrating ductal carcinoma tissues (panels B,D,F,H) were reacted with monoclonal antibodies for CA15-3 (panels A,B), integrin $\alpha 3$ subunit (panels C,D), laminin5 (panels E,F), or integrin $\beta 4$ (panels G,H). Bound antibodies were visualized as described in Methods and sections were counterstained with hematoxylin.

II. CONCLUSIONS

A. The results of the past year suggest that the integrin $\alpha 5 \beta 1$ may not play a significant role in the regulation of mammary epithelial cell proliferation. This conclusion is based on the lack of staining for $\alpha 5 \beta 1$ in either normal or cancer tissue as well as our examination of cell cycle kinase activity in response to RGD (GRGDS) peptides in primary cultures of normal cells as well as a variety of breast cancer cell lines. Although soluble RGD peptides binding to $\alpha 5 \beta 1$ may not be relevant to mammary epithelial cell growth, the examination for a possible signaling pathway directly linking integrins with cell cycle regulation remains an intriguing avenue of research. Our data suggests that other integrins bind RGD peptides in breast cancer cell lines which lead to changes in the activity of cell cycle associated kinases (cdc2 and cyclin A). We will characterize these events further using RGD peptides which bind preferentially to vitronectin receptors ($\alpha v \beta 3$, $\alpha v \beta 5$) or to fibronectin receptors ($\alpha 5 \beta 1$, $\alpha v \beta 6$, $\alpha v \beta 1$) which are available from Life Technologies. We will also carry out immunoprecipitations in combination with Western blot techniques to identify specific cyclin/cdk complexes. This is particularly relevant to determine which cdk(s) are associated with the changes in kinase activity observed for cyclin A.

In fibroblasts, a possible link between integrins and MAP kinase has been made via focal adhesion kinase (FAK). Recently, FAK overexpression in MDCK epithelial cells promoted anchorage-independent growth (survival) in the absence of MAP kinase activation (Frisch, S.M. et al., 1996). Thus, epithelial cells appear to differ from fibroblasts in their regulation of MAP kinase by FAK. While the authors attributed these results to an inhibition of apoptosis, it is also possible that FAK overexpression results in constitutively active cell cycle kinase(s) in the absence of cell adhesion. Therefore, we will include analyses of FAK activity in our cell cycle kinase assays to determine if there are connections between these two kinase systems. FAK activity will be assayed by looking at autophosphorylation of FAK as well as using commercially available kits for tyrosine kinases (Boehringer Mannheim cat # 1-534-505; Life Technologies cat # 13154-018). Monitoring of FAK activity should provide a clue as to the role of the focal adhesion complex in signaling regulators of cell cycle dependent kinases. Since overexpression of cyclin D has been reported to be associated with breast cancer (Courjal, R., et al., 1996), we will look for possible links between integrins and cyclin D associated kinase activity. If we can demonstrate an integrin-mediated regulation of the cell cycle in normal cells, we may be able to utilize some of the cancer cell lines based on their integrin profile for further characterization.

B. We have characterized the integrin repertoire on 12 different breast carcinoma cell lines as well as on one batch of normal mammary epithelial cells. The most prominent integrin characterized to date is $\alpha 3 \beta 1$ which is expressed at relatively high levels on 10 of the 12 cell lines and most strongly on normal cells. Lower and more variable expression was observed for $\alpha v \beta 5$, $\alpha 2 \beta 1$, $\alpha 5 \beta 1$, $\alpha 4$, and $\alpha 6$. None of these cells seem to express significant levels of $\alpha v \beta 3$ or $\beta 4$. Variable levels of expression was also observed for E-cadherin and CA 15-3. We will expand our characterization of integrin expression

to other receptors such as $\alpha v \beta 6$, $\alpha v \beta 1$, and $\alpha 1 \beta 1$. Towards this aim we have recently obtained a monoclonal antibody to $\alpha v \beta 6$ from Dr. Dean Sheppard (University of California at San Francisco).

The integrin $\alpha 3 \beta 1$ is one extracellular matrix receptor that is expressed on both normal and tumor cells. While this integrin appears to be primarily a receptor for laminin 5, it has also been shown to mediate cell attachment to laminin 1, fibronectin, and type I collagen. Binding to fibronectin has been shown to be sensitive to soluble RGD peptides. We will therefore be particularly interested in characterizing the $\alpha 3 \beta 1$ - mediated adhesion to its various ligands.

Since it is unclear from the data generated this past year which integrin(s), if any, are involved in regulating cell growth of mammary epithelial cells, we will also focus on characterizing normal mammary epithelial cell growth in response to different extracellular matrix proteins (fibronectin, collagen type I, collagen type IV, laminin 1, and laminin 5). These proteins will either be purified as described previously (Wayner and Carter, 1987, Wayner et al, 1988, Wayner et al, 1991) or obtained from commercial sources (Life Technologies, Gaithersburg MD). Laminin 5 will be purified by capturing the material to 96 or 48 well plates with monoclonal antibodies (Xia Y.P., et al 1996). We will look for increases in cell number, increases in thymidine incorporation, and changes in cell cycle kinase activity. A matrix-specific response would suggest that specific integrins are involved in cell growth regulation. We will also utilize monoclonal antibodies to specific integrins to see if clustering or monovalent interactions stimulate cell growth and/or cell cycle kinase activity.

C. We have encountered difficulties in isolating primary normal and tumor cells in sufficient quantities for biochemical characterization. We will continue efforts to improve current methods of cell isolation and growth. During this method development, we will continue to purchase normal mammary epithelial cells from Clonetics Corp. (San Diego, CA).

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V. Appendices

A. Bibliography of publication and meeting extracts.

NONE

B. List of personnel:

1. Elizabeth Wayner, Ph.D., Principal Investigator
2. Richard Tamura, Ph.D., Associate Scientist
3. Wendy Pabich, Research technician I
4. John Fisher, Research technician II



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